Effects of Boiling on the IgE-Binding Properties of Tropomyosin of Shrimp (*Litopenaeus vannamei*)

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ABSTRACT: The thermal stability and IgE binding of raw and boiled shrimp extracts and the tropomyosins (TM) have not been reported. In this study, we compare the stability of raw and boiled shrimp TM of *Litopenaeus vannamei* and evaluate how boiling may alter the allergenicity of *L. vannamei*. Extracts were prepared from raw and boiled shrimp and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis. The IgE-binding of the extracts was determined by western-blot and competitive inhibition enzyme-linked immunosorbent assay (iELISA). The TM was then purified from raw and boiled shrimp, the secondary structures analyzed by circular dichroism (CD) spectroscopy, and the IgE binding compared by slot blot analysis. The soluble protein content decreased and the higher molecular weight proteins increased in the extracts from boiled versus raw shrimp. Similar IgE binding characteristics were seen by extracts when using western blot analysis. Although iELISA results showed that extracts from raw shrimp bound higher IgE than extracts from boiled shrimp, dot-blot assay demonstrates higher IgE binding to purified TM from boiled shrimp than raw shrimp. The purified TM had a typical alpha-helical secondary structure and the stability of boiled TM was lower than that of raw TM. Extracts from boiled shrimp produce lower IgE binding than extracts from raw shrimp, which suggest that boiling can be used as a tool in attempting to reduce shrimp allergenicity. However, the purified TM from boiled shrimp, which shows enhanced IgE binding over that of raw shrimp, may be a more effective antigen in diagnosing shrimp allergy through immunoassay.

Keywords: boiling, CD-spectrum, *Litopenaeus vannamei*, processing, shrimp allergy, tropomyosin

Introduction

Shellfish allergy is a long-lasting disorder, usually persisting throughout life, and is often associated with severe reactions, including life-threatening anaphylaxis (Steensma 2003). The prevalence of immediate-type shellfish allergy is higher in areas with high shellfish intake. About 16 y ago, it was estimated that approximately 250000 Americans experienced allergic reactions to shellfish (Musmand and others 1993). A more recent survey by Sicherer and others (2004) found that 1 in 50 Americans had shellfish allergy. Shellfish is the number one cause of food allergy in adults in the United States and is responsible for the majority of emergency department visits (Clark and others 2004).

A large variety of shellfish are used for human consumption. *Litopenaeus vannamei* is the most widely cultured shrimp species in the world, more than 900000 tons are consumed annually (Ayuso and others 2008). Crustacean shellfish, such as shrimp and crab, are among the major allergenic foods (FAO/WHO 2001). Research has demonstrated that the major allergen of shellfish is tropomyosin (TM), a myofibrillar protein composed of 2 identical subunits with molecular masses of 35 to 38 kDa (Shanti and others 1993; Leung and others 1998). A number of IgE-binding epitopes have been identified in this molecule, though they may vary from one allergic individual to another (Shanti and others 1993; Motoyama and others 2007). Patients with shrimp allergy often exhibit allergic symptoms to a variety of seafood such as crabs and clams. TM has been described as an important food allergen in shrimp, lobster, crab, oysters, squid, and so on. Allergic reactions to TMs are often cross-reactive, which may be explained by the highly conserved amino acid sequences (Lehrer and others 2003).

Cooking processing may destroy existing epitopes on a protein or may generate new ones (neoallergen formation) as a result of change in protein conformation. (Maleki and others 2000; Lehrer and others 2003; Taylor 2008). Shrimp is often consumed after certain degree of heat (such as boiling). An understanding of the allergenic properties of shrimp as affected by the cooking process is critical especially for shrimp allergic individuals. The aims of the study were to compare the stability of raw and boiled shrimp extracts or TM of *L. vannamei* and evaluate how the cooking process may alter the allergenicity of *L. vannamei*.

Materials and Methods

Shrimp and human serum

L. vannamei was purchased live at New Orleans market (La., U.S.A.). After washing, shrimp muscle was obtained and immediately used for experiment. The sera (PL-BH, PL-RE, JL, LW, and KW) used in this study with specific IgE antibody to shrimp TM were obtained from 5 patients with shrimp allergy in New Orleans, La., U.S.A. Control sera were obtained from 2 normal subjects who had shown no adverse reactions to any foodstuff. Blood samples were collected after informed consent, and the Institutional Review Board approved the study. These sera were frozen at −80 °C until use.

Reagents

Protein standards for SDS-PAGE were from Bio-Rad (Richmond, Calif., U.S.A.). Protein standards for western-blot were from

New England BioLabs (Beverly, Mass., U.S.A.). Horseradish peroxidase (HRP)-labeled rabbit antihuman IgE was from Sigma (Sigma, The Woodlands, Tex., U.S.A.). HRP-labeled goat anti-rabbit IgG and 3, 3′-diaminobenzindin tetrahydrochloride (DAB) were from Sigma (Sigma, The Woodlands). Rabbit antimud crab TM polyclonal antibodies were prepared in our laboratory (Liang and others 2008). Bovine serum albumin (BSA) was from Sigma-Aldrich. ReadyStrip[TM] IPG strips (11 cm, pH 4 to 7) were from Bio-Rad. Biotin labeled rabbit anti-human IgE, Strept Avidin HRP, SureBlue[TM] TMB Microwell peroxidase substrate were from KPL (Gaithersburg, Md., U.S.A.).

Preparation of shrimp extracts, purification of TM, and protein content determination

The same amount of starting material (raw shrimp) was used for preparation of extracts or purified TM. Shrimp TM was prepared by the method of Shimakura and others (2005) with some modifications. In brief, boiled shrimp extracts were prepared after the raw material was boiled for 10 min in water. Extracts from raw and boiled shrimp were ground into slurry using a grinder and then freeze-dried. The samples were solubilized by adding 10 g of the shrimp powder to 500 mL of 50 mM Tris-HCl (pH 7) followed by sonication and centrifugation at 12000 rpm for 30 min. The extracted proteins were divided into aliquots and stored at −20 °C. Afterward, TM was purified from the extracts by centrifugation at $12000 \times g$ for 30 min; the supernatant was partially purified by ammonium sulfate fractionation at 25%, 50%, 75%, and 100% saturation. The pellet was collected by centrifugation at $12000 \times g$ for 30 min and dissolved in 50 mM Tris-HCl (pH 7). The precipitation from ammonium sulfate at 50% saturation was loaded on 2.5×20 cm DEAE-support fast flow column (weak anion exchange support, Bio-Rad Lab.) and eluted with a 300 to 600 mM NaCl linear gradient in 50 mM Tris–HCl, pH 7; the collection then loaded on 2.5×20 cm high S-support fast flow column (strong cation exchange support, Bio-Rad Lab.) and eluted with a 50 to 1000 mM NaCl linear gradient in 50 mM Tris–HCl, pH 6.5; the collection then loaded on 2.5×20 cm high Q-support fast flow column (strong anion exchange support, Bio-Rad Lab.) and eluted with a 200 to 1000 mM NaCl linear gradient in 50 mM Tris–HCl, pH 8.4. The shrimp TM with high purity was used for experiments or stored at −80 °C until used.

The protein concentrations of crude extracts were determined by the method of Lowry and others (1951) and that of purified shrimp TM were measured the absorbance at 280 nm (UV-VIS Spectrophotometer, Shimadzu, Japan). The theoretical extinction coefficient of 2400 for TM is used for the CD spectrums.

SDS-PAGE and western-blot analysis

The proteins extracted in the raw and boiled shrimp were separated by SDS-PAGE on 4% to 20% 12-well Tris-Glycine gel (Invitrogen, Carlsbad, Calif., U.S.A.), and were stained by GelCode Blue Stain Reagent (Thermo Scientific, Waltham, Mass., U.S.A.). For western blotting, the proteins were transferred with iBlot[TM] gel transfer stacks (Invitrogen) to a PVDF membrane. Membranes were preblocked for 1 h at room temperature (RT) in 5% blotto (5% dry milk made in phosphate buffered saline and 0.05% tween-20; PBST).

For IgG binding, the primary antibodies, anti-crab TM polyclonal antibodies (prepared in our laboratory), were diluted (1 : 30000) in 5% blotto and incubated with the PVDF membrane for 1 h at RT. The secondary antibodies used were HRP-labeled goat anti-rabbit IgG (Sigma) at 1 : 100000.

Serum IgE binding was performed in a similar way, with blocking at RT for 30 min in 2% blotto and then incubating with 1 : 5 or 1 : 10 dilution of serum from shrimp allergic individuals or pooled sera from 5 shrimp allergic individuals diluted 1 : 5 and incubated with membranes for 1 h at RT. The secondary antibodies used were HRP-conjugated rabbit anti-human IgE (Sigma) at 1 : 10000.

Both for IgG and IgE binding, the secondary antibodies were diluted in 2% blotto and incubation time was 30 min at RT. Following secondary antibody incubation, membranes were washed extensively with PBST and incubated with ECL substrate (Amersham Bioscience Corp, Piscataway, N.J., U.S.A.) according to manufacturer's instructions. The signal was measured using CCD camera system (Fuji Photo Film Co., Ltd., Duluth, Ga., U.S.A.).

Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out according to a published method (Yu and others 2003). Isoelectric focusing (IEF) in the 1st dimension was done (Protein IEF Cell, Bio-Rad) using ReadyStrip[TM] IPG strips with an immobilized pH gradient (4 to 7), 11 cm, containing 8 M urea and 10 mM dithiothreitol. Gel strips were rehydrated overnight in 185 μL IEF buffer (8 M Urea, 2 mM TBP, 4% CHAPS, 0.2% Carrier ampholytes, 0.0002% Bromophenol Blue). A sample of 200 μg protein was applied and separated at 8000 V and 2 mA for 6 h. After subsequent equilibration steps in buffer I (6 M urea, 20% glycerol, 50 mM Tris-HCl, pH 8.8, 2% SDS, 0.13 M DTT) and buffer II (6 M urea, 20% glycerol, 50 mM Tris-HCl, pH 8.8, 2% SDS, 2.5% iodoacetamide), SDS-PAGE was performed at 100 V and 50 mA using Criterion[TM] precast gel (Bio-Rad). The isoelectric point (pI) and molecular mass were determined using an IEF test mixture (pH 4 to 7, Bio-Rad) and protein standard (Bio-Rad) as marker. The 2nd dimension gel was stained with Sypro Ruby (Bio-Rad).

IgE inhibition ELISA

IgE inhibition ELISA analysis were performed by the methods described previously (Engvall and Perlmann 1971; Carnés and others 2007) with a slight modification. For ELISA cross-inhibition studies, the sera from 5 patients displaying high IgE reactivity to raw shrimp extracts were used. Immunlon 4BX microtiter plates (Costar, Cambridge, Mass., U.S.A.) were coated for 16 h at 4 °C with 50 μL of extracts from raw shrimp (0.02 μg/μL) in 50 mM NaHCO$_3$, pH 9.5, with blocking at RT for 30 min in 2% blotto and then probed 1 h at 37 °C with aliquots of the serum previously incubated (RT for 1 h) with different concentrations of raw shrimp extracts and boiled shrimp extracts. BSA was used as the negative control. After washing with PBST, pH 7, the plates were incubated for 1 h at 25 °C with biotin-labeled goat anti-human IgE Abs (KPL) (1 : 4000) and Strept Avidin HRP (1 : 800) (KPL), then color development was performed using SureBlue TMB Microwell peroxidase substrate (KPL), the OD was measured at 450 nm using an ELISA plate reader (Sunrise Tecan, Boston, Mass., U.S.A.). All assays were performed in triplicate.

Circular dichroism (CD) spectroscopy

To determine the circular dichroism (CD) spectra of raw TM and boiled TM, 300 μL of each TM was placed into a 1 mm pathlength cuvette and maintained at 25 °C. The change in far-UV CD spectrum from 185 to 250 nm was then measured using a JASCO 815 spectropolarimeter equipped with a Peltier temperature control system (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The α-helix content was calculated from the molar ellipticity at 222 nm. The CD spectrum of TM was determined with a concentration of

0.5 μM at 25 °C for 7 times. Thermal transition of 0.5 μM TM in water was monitored, at 222 nm in a temperature range between 25 and 95 °C with a temperature slope of 10 °C/min (Ambler and others 1974; Nakamura and others 2006). Molar ellipticity was calculated according to the published methods (Becktel and Schellman 1987; Greenfield 1996; Verdino and Keller 2004). The cooling graph was recorded after a delay time of 5 min.

Dot-blot

Dot-blot was performed by the method described previously (Nakamura and others 2006) with a slight modification. The subjects' sera were diluted 1 : 5 or 1 : 10. The ECL western blotting detection reagent (Amersham, Buckinghamshire, U.S.A.) was used to detect the enzyme reaction. The higher intensity of spot indicates an increase in the amount of specific IgE bound to TM.

Results and Discussion

Total protein extraction and 1-D and 2-D electrophoreses

The SDS-PAGE profiles of the shrimp extracts showed multiple protein bands ranging from 10 to 200 kDa. Both extracts had a prominent band at approximately 38 kDa that was similar to the molecular weight of TM. The 38 kDa protein is more prominent in extracts of boiled shrimp as compared to raw shrimp (Figure 1A). The 38 kDa proteins from raw and boiled shrimp were recognized by a rabbit anti-TM antibody in western-blot studies (data not shown).

To identify the IgE-binding proteins in shrimp extracts, crude extracts of raw shrimp and boiled shrimp were used as antigen and analyzed by western blot. Sera samples from 5 shrimp allergic patients all reacted with the crude cooked extract (Figure 1). Though

more than 3 protein bands with molecular masses ranging from 24 to 90 kDa could be identified as reacting with the shrimp-allergic patients' sera, the predominant band, was 38 kDa and was recognized by all 5 patients. And it was shown that extracts from both raw shrimp and boiled shrimp retained an IgE-binding protein at 38 kDa. The remaining IgE-binding components with various molecular masses, such as 24 and 78 kDa, however, were detected at lower intensity. No IgE binding protein was detected with pooled normal negative control sera (data not shown). The results found that all the 5 sera (100%) had positive IgE-binding to the protein. The protein of 38 kDa may be TM, the well known major allergen of crustaceans (Leung and others 1994).

Proteins were also separated by means of 2-D electrophoresis, and either stained with Gel Code or Sypro Ruby for total protein analysis. Figure 2 shows the 2-D electrophoresis results of extracts from raw and boiled shrimp. The major change after boiling was the modification and loss of the proteins of low molecular weight and the increased apparent yield of the proteins of high molecular weight in extracts from boiled shrimp. The proteins of high molecular weight in extracts from boiled shrimp may be the results of the aggregation of proteins and chemicals (Hefle 1996).

Comparison of IgE binding to crude extracts from raw and boiled shrimp using inhibition ELISA

The IgE-binding of extracts from raw and boiled shrimp was assessed using inhibition ELISA. Briefly, raw shrimp extracts were coated on 96-well microtitre plate. Various concentrations of either raw or boiled shrimp extracts were diluted with serum from shrimp-allergic patients and bound IgE antibodies were detected using biotin labeled anti-IgE antibody directed against human IgE and Strept Avidin HRP. The inhibition rate of extracts from raw shrimp (Figure 3A) was approximately 8-fold higher than to that of extracts from boiled shrimp (Figure 3B) as assessed by the protein concentration needed to exert 50% inhibition. Boiling may cause lower IgE binding because some of the IgE-binding epitopes may be masked as a result of protein-chemical cross-linking (Nakamura and others 2005). Alteration in protein structure (by boiling) also can lead to epitope destruction or modification thereby decreasing the IgE binding (Paschke and Besler 2002).

Establishment of an efficient purification step

Protein components of the crude extracts from *L. vannamei* were analyzed by SDS-PAGE (Figure 4A, lane 1). TM was further purified to homogeneity by 50% ammonium sulfate fractionation and column chromatography (DEAE fast flow column, high S sepharose fast flow column, and high Q sepharose fast flow column). The molecular mass of the purified protein was approximately 38 kDa (Figure 4A, lane 5). The identity of the purified TM was further confirmed by western-blot with pooled sera from 5 shrimp allergic patients (Figure 4C for raw shrimp, Figure 4D for boiled shrimp) and rabbit anti-crab TM polyclonal antibody (data not shown).
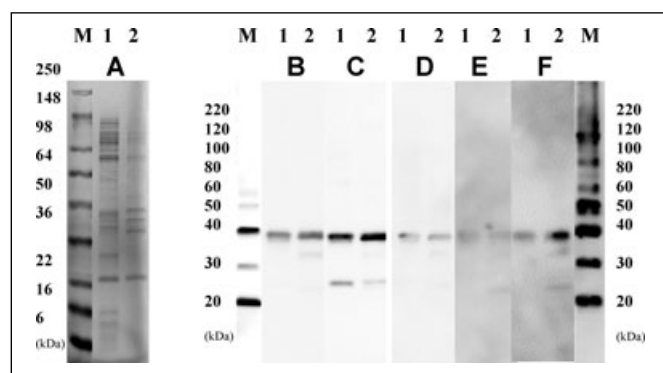

Figure 1—SDS-PAGE analysis (A) of the extracts from shrimp and western-blot analysis of IgE binding with patient sera (B: PL-BH, C: PL-RE, D: JL, E: LW, and F: KW). Positions of molecular mass standards (M) for SDS-PAGE and western-blot were labeled on the left and right. Gels were stained by GelCode Blue Stain Reagent (Thermo Scientific). Lane 1, raw shrimp extracts; lane 2, boiled shrimp extracts.
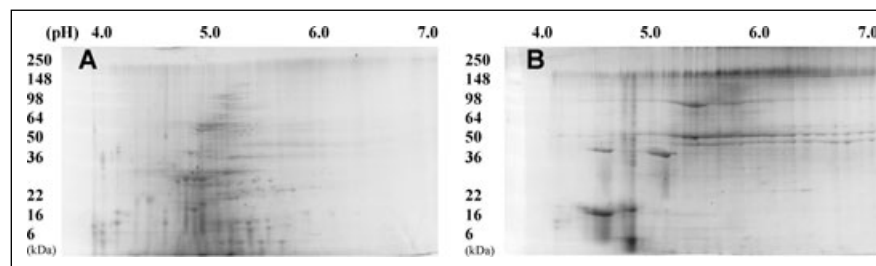

Figure 2—Two-dimensional proteomics map (11 cm, pH 4 to 7) for extracts from raw shrimp (A) and boiled shrimp (B) labeled with Sypro Ruby. Molecular weight standards (M) are shown at left. Major change was the degradation of proteins of low molecular weight and yielding of proteins of high molecular weight in extracts from boiled shrimp.

Analysis secondary structure of TM by CD spectroscopy

The secondary structure of TM purified from raw shrimp and boiled shrimp was analyzed by CD spectroscopy and the far-UV spectra are shown in Figure 5A. The spectrum exhibits
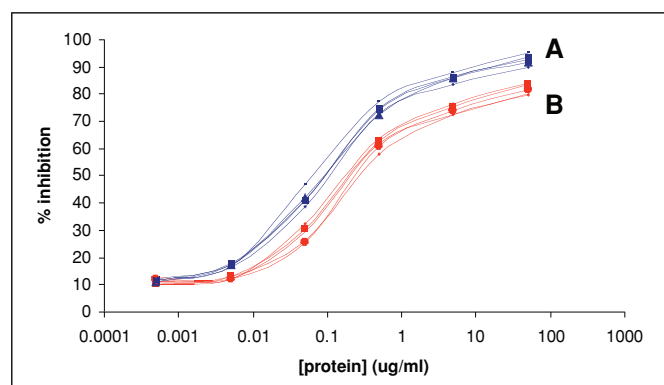
Figure 3 — IgE-binding properties of extracts from raw shrimp (A) compared with boiled shrimp (B) using inhibition ELISA with different patient sera. The results are given as the means of triplicate measurements. Five patients' sera: (■), PL-BH; (△), PL-RE; (□), JL; (·), LW; (▲), KW, respectively. Coated sample was raw shrimp extracts and inhibitor was raw shrimp extracts and boiled shrimp extracts, respectively.
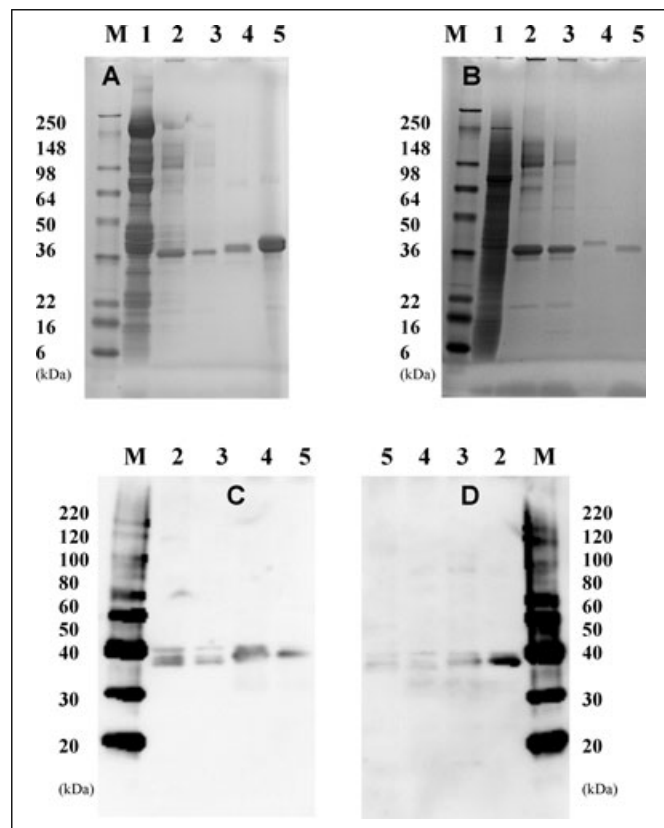

Figure 4 — SDS-PAGE and western-blot analysis of pooled sera from 5 shrimp allergic patients for various purified TM fractions from raw shrimp extracts (A, C) and boiled shrimp extracts (B, D). Positions of molecular mass standards (M) for SDS-PAGE and western-blot were labeled on the left and right. Lane 1, extracts in Tris-buffer; lane 2, 50% ammonium sulfate cut; lane 3, fractions from the DEAE fast flow column; lane 4, fractions from the high S sepharose fast flow column; lane 5, fractions from the high Q sepharose fast flow column.

minimums at 222 and 208 nm, which are typical for alpha-helical secondary structure such as what is found in TMs (Nakamura and others 2006) Additionally, the minimum at 222 nm is greater than that of at 208 nm, which is indicative of a coiled-coil structure again corroborating the structure of TMs (Nakamura and others 2006).

To analyze and compare the thermal stability of TM, the secondary structure was analyzed at different temperatures. The wavelength at 222 nm was monitored throughout as an indicator of loss of alpha-helical secondary structure and thus, stability of the proteins (Greenfield 1996). The graph of the far-UV spectra at temperatures from 25 to 95 °C is shown in Figure 5B. The results clearly indicate unfolding of the allergen at a calculated temperature of 45 °C. The reduction in the ellipticity throughout the spectrum is not due to the protein precipitating, the protein was able to refold when cooled to 25° C. The thermal stability of the proteins was compared by monitoring the alpha helical content of raw to boiled TM at the wavelength 222 nm (Nakamura and others 2006). As shown in the graph in Figure 5, the thermal stability for boiled TM and raw TM is quite the same.

Comparison of IgE binding to purified TMs from raw and boiled shrimp using a dot-blot assay

The IgE binding of a shrimp-allergic patient to purified TMs was analyzed by dot blot. As shown in Figure 6, boiled TM showed consistent IgE binding with 5 patients' sera even at a protein concentration of 32 ng, whereas the IgE binding to raw TM weakened with the dilution of the protein almost disappearing at the concentration of 32 ng. No IgE binding was observed under the
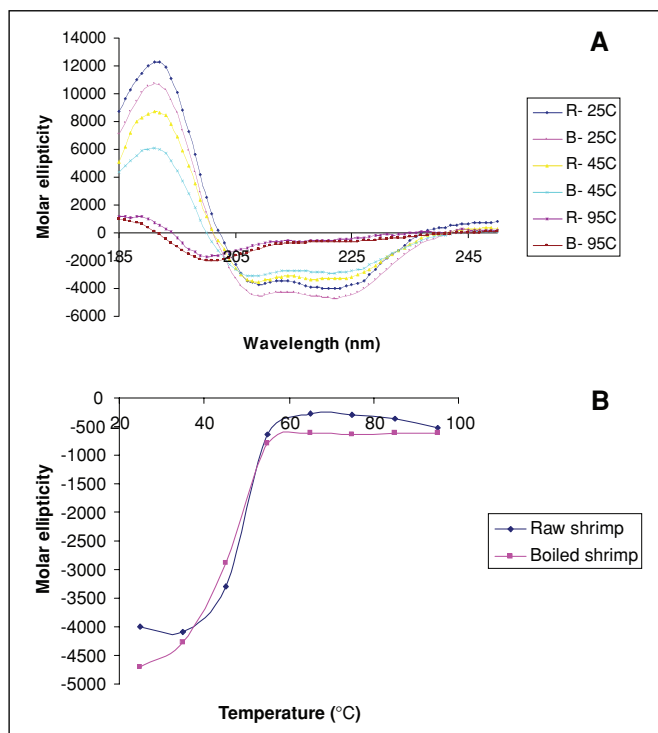
Figure 5 — CD-spectrum of TM in water (0.5 μmol/L). The secondary structure of the purified TM was confirmed by CD analysis showing minima at 222 and 208 nm, typical for alpha-helical protein. (A) CD spectra showing the effects of heating on the secondary structures of TM. No significant conformational changes at 25 °C, but an increased dichroism and a loss of the minimum at 222 nm were monitored 45 and 95 °C. (B) Thermal transition curve of TM recorded at 222 nm. An identical curve was obtained when the solution was cooled again.
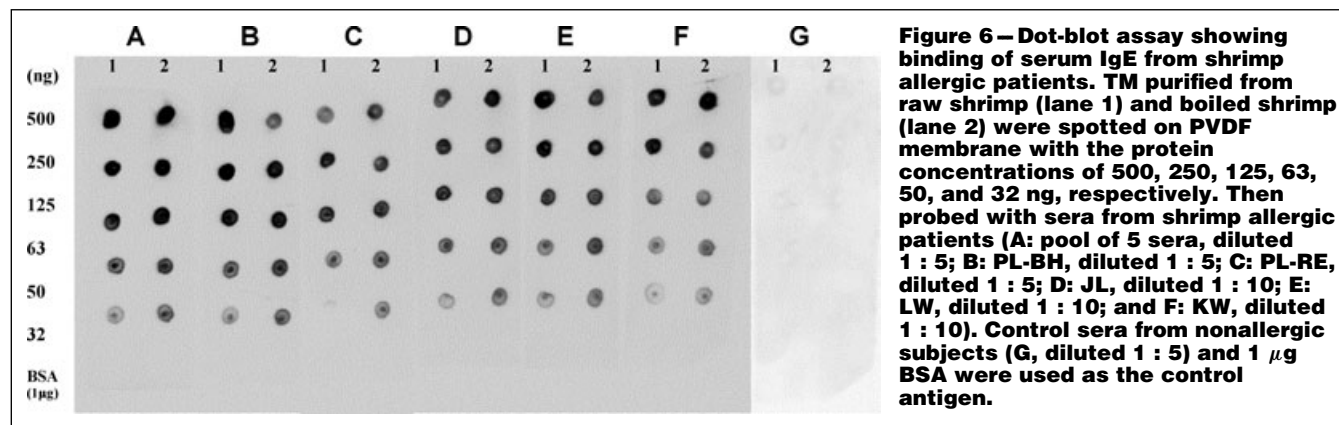
Figure 6 – Dot-blot assay showing binding of serum IgE from shrimp allergic patients. TM purified from raw shrimp (lane 1) and boiled shrimp (lane 2) were spotted on PVDF membrane with the protein concentrations of 500, 250, 125, 63, 50, and 32 ng, respectively. Then probed with sera from shrimp allergic patients (A: pool of 5 sera, diluted 1 : 5; B: PL-BH, diluted 1 : 5; C: PL-RE, diluted 1 : 5; D: JL, diluted 1 : 10; E: LW, diluted 1 : 10; and F: KW, diluted 1 : 10). Control sera from nonallergic subjects (G, diluted 1 : 5) and 1 μg BSA were used as the control antigen.

same conditions with the control serum. Therefore, the results of Figure 6 indicate that boiled TM has enhanced specific IgE-binding over that of raw TM. Results were not so unexpected to most people in the United States eat cooked shrimp. Therefore, it makes sense that allergic patient IgE recognizes the boiled TM better. Boiled TM may have undergone protein–protein interactions (especially aggregation) during thermal treatment to cause enhanced IgE binding (Mine and Yang 2008). Furthermore, unmasking of boiled TM may result in IgE epitopes being more surfaces accessible (Aalberse 2000).

Conclusions

Shrimp is one of the major allergenic foods and is often consumed after certain degree of cooking (such as boiling). An understanding of the allergenic properties of shrimp as affected by the cooking process is critical especially for shrimp allergic individuals. This study examined the effect of boiling on the allergenicity of shrimp and compared the allergenicity and structural stability of TM extracted from both raw and boiled shrimp.

Boiled shrimp extracts showed lower IgE binding than raw shrimp extracts. Boiling may alter shrimp extracts in a manner that may permit masking of allergenic epitopes thereby reducing allergen recognition and therefore potentially altering allergenicity of the food, which suggest that boiling can be used as a tool in attempting to reduce/eliminate food allergens. The thermal stability of TM purified from boiled shrimp was same as that of TM purified from raw shrimp. And IgE-binding activity of boiled TM was stronger than to that of raw TM. Boiled TM may have undergone protein–protein interactions (especially aggregation) during thermal treatment to cause enhanced IgE binding and unmasking of IgE epitopes to have more accessible surfaces. The use of boiled TM seems to be more effective in diagnosing seafood allergy.

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